Enantioselective Fluorogenic Assay of Acetate Hydrolysis for Detecting Lipase Catalytic Antibodies

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An enantioselective fluorogenic assay for the kinetic resolution of chiral alkyl acetates is demonstrated with 7-(3-acetoxybutoxy)-2*H*-1-benzopyran-2-ones (*R*)- and (*S*)-**4** or 7-(3-acetoxy-2-methylpropoxy)-2*H*-1-benzopyran-2-ones (*R*)-**4** and (*S*)-**6**. The alcohols released by hydrolysis of these acetates are oxidized by horse-liver alcohol dehydrogenase to unstable β-(aryloxy)carbonyl compounds, which undergo β-elimination of the strongly fluorescent product umbelliferone (= 7-hydroxy-2*H*-1-benzopyran-2-one; **3**) ($\lambda_{em} = 460 \pm 20$ nm, $\lambda_{ex} = 360 \pm 20$ nm). Enantioselectivities are calculated from the reaction rates for each enantiomeric acetate. For a series of representative lipases, the reactivities and enantioselectivities under preparative conditions are predicted accurately. This highly sensitive enantioselective assay detects as little as 10 μg/ml of hydrolytic enzyme, can be carried out in 96-well microtiter plates, and is compatible with cell-culture media. It is, therefore, suited for screening libraries of antibodies for enantioselective lipase catalytic antibodies.

Introduction. – A broad variety of biocatalysts such as enzymes isolated from various sources and catalytic antibodies are used today for producing chiral synthetic intermediates [1]. Kinetic resolution of chiral racemic esters using lipases and esterases represents the most widely used biotransformation. Although a broad palette of such enzymes are available, not all racemic esters can be resolved, and new esterases capable of resolving simple esters would be welcome to complement the current arsenal.

One of the most efficient methods for discovering new biocatalysts consists in screening libraries of possible catalysts for activity and enantioselectivity. This approach requires the availability of a simple assay for catalysis applicable in high throughput format, typically in 96-well microtiter plates. If the catalyst's concentration in each sample is very small, the catalysis assay must in addition be highly sensitive. This situation is typically realized when assaying catalytic antibodies in cell culture. Sensitive catalysis assays have been reported based on product-specific detection systems using either a product-specific antibody (*cat*-ELISA) [2], a biotin tag [3], a DNA tag [4], or a fluorescent acridone tag [5].

We have recently shown that fluorogenic substrates are optimally suited for detecting catalytic antibodies directly in cell culture [6]. However, fluorogenic or chromogenic substrates for esterase-activity screening are normally esters of aromatic phenols [7]. Such esters are chemically activated for hydrolysis, which implies that a biocatalyst isolated for its hydrolytic activity on such esters might not catalyze the hydrolysis of the more stable aliphatic esters, which are the synthetically relevant substrates.

We have investigated several possibilities to make the hydrolysis of aliphatic esters a fluorogenic reaction. One of our approaches involves a general fluorogenic assay for

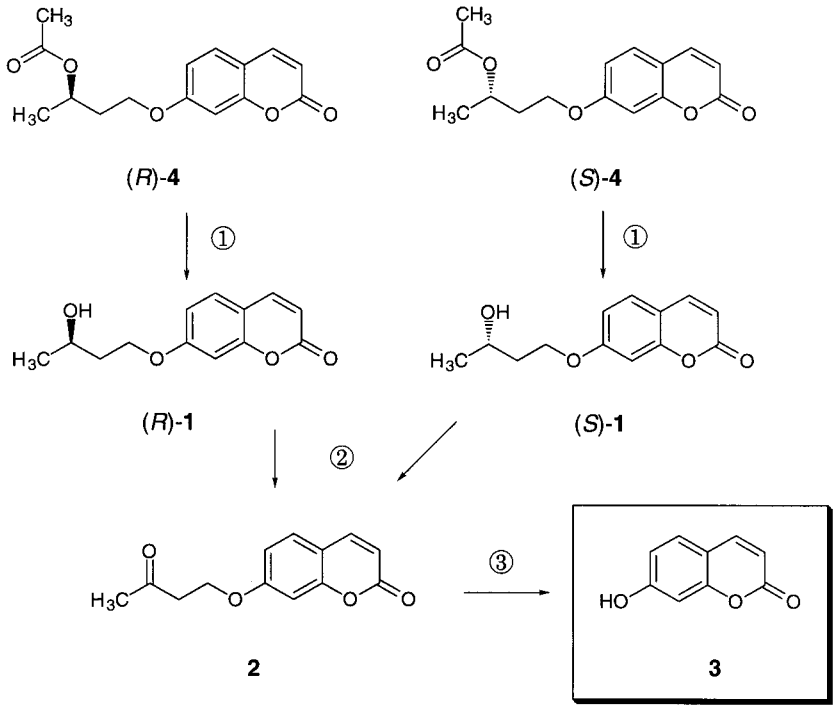
catalysis based on an antibody sensor [8]. The method was demonstrated by the example of an aliphatic ester and could potentially be applied to chiral esters. Herein we report an alternative and simpler approach to a practical enantioselective fluorogenic assay for the hydrolysis of chiral acetates. The procedure is based on a sequence of two coupled biocatalytic steps, involving horse-liver alcohol dehydrogenase (HLDH) and bovine serum albumin (BSA), that converts a pair of enantiomeric aliphatic alcohols formed by acetate cleavage to a fluorescent product. This enantioselective assay is highly sensitive and compatible with cell-culture media, and is, therefore, optimally suited for high throughput screening of antibody libraries for new lipase catalytic antibodies [9].

Results and Discussion. – The enantiomeric alcohols (*R*)- and (*S*)-**1** can be used as fluorogenic substrates for alcohol dehydrogenases [10]. Their oxidation releases ketone **2**, which is unstable and undergoes β-elimination of the strongly fluorescent product umbelliferone (**3**) under catalysis by BSA. The same strategy provides a stereoselective fluorogenic assay for aldolases [11]. By contrast to most fluorogenic substrates such as esters and ethers or aromatic fluorophores, alcohols (*R*)- and (*S*)-**1** are chemically unbiased in their reactivity and chiral environment and are, therefore, representative of 'normal' synthetic substrates. We now show that the corresponding acetates (*R*)- and (*S*)-**4** serve as fluorogenic substrates for an indirect assay of esterase activity when their hydrolysis is carried out in the presence of an alcohol dehydrogenase and BSA (*Scheme 1*). Isomeric primary alcohols (*R*)- and (*S*)-**5** are similarly formed from acetates (*S*)- and (*R*)-**6**, respectively, and oxidized to release fluorescent umbelliferone (**3**; *Scheme 2*).

The ideal setup for a kinetic resolution assay should involve two measurements with racemic acetate substrate and each of two different alcohol dehydrogenases oxidizing enantioselectively either the (*R*)- or the (*S*)-alcohol. While two alcohol dehydrogenases oxidized (*S*)-**1** and (*S*)-**5** with very high enantioselectivities, no dehydrogenase was found to oxidize the (*R*)-configured alcohols with high enantioselectivities. However, we found that HLDH (NAD-dependent) oxidized all four alcohol substrates with satisfactory rates (*Table 1*). We, therefore, turned to a setup involving a pair of assays with enantiomerically pure (*R*)- or (*S*)-substrates in the presence of HLDH as a single enzyme.

The stability of our acetate substrates was first tested under different pH and buffer conditions with and without BSA. We use BSA as a simple but efficient additive to catalyze the secondary decomposition of the β-(aryloxy)carbonyl compounds produced by oxidation of the alcohols, which liberates the fluorescent product umbelliferone. Secondary acetates **4** showed good stability under dehydrogenase-assay conditions (pH 8.8, 2 mg/ml BSA). Similar control experiments in hybridoma cell-culture media, which is a phosphate-buffered medium containing calf serum, showed that acetates were also stable under these conditions. By contrast, primary acetates **6** were completely unstable at pH 8.8 with or without BSA, or in cell-culture media. Satisfactory stability for acetates **6** was obtained in pH 7.2 *Bis-tris* buffer, with and without BSA. Under these conditions, an excellent fluorescence signal was obtained due to the unexpectedly high reactivity of the corresponding alcohols (*R*)- and (*S*)-**5** with HLDH.

Scheme 1. *Fluorogenic Esterase Assay with Secondary Acetates (*R*)- and (*S*)-***4***: Esterase or lipase reaction* (①) *is followed by oxidation* (②: HLDH, NAD^+) *and β-elimination* (③: BSA, pH > 7) *of the fluorescent product umbelliferone* ***3***



A series of thirty different esterases and lipases was assayed in the presence of HLDH/NAD^+ and each of the four chiral acetates (*R*)-**4**, (*S*)-**4**, (*R*)-**6**, and (*S*)-**6** by following the rate of release of umbelliferone (**3**) by fluorescence in microtiter plates[1]). An assay with the corresponding alcohols (*R*)-**1**, (*S*)-**1**, (*R*)-**5**, and (*S*)-**5** and HLDH/NAD^+ was used as reference for the maximum observable rate. An assay with each acetate and HLDH/NAD^+ but without esterase or lipase was used as background reference. With the most active lipases, we checked that the net rate over background was proportional to the lipase concentration. This established that the apparent rate of umbelliferone release was directly proportional to the rate of acetate hydrolysis. Thus, a relative rate of acetate hydrolysis could be calculated for each acetate/enzyme pair by dividing the net rate of umbelliferone release over background by the reference maximum rate obtained with the corresponding alcohol. Predicted enantiomeric excesses for released alcohol were then calculated from relative rates (*R*)- and (*S*)-acetates (*Figs. 1* and *2*, and *Table 2*).

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[1]) The following lipases and esterases gave no reaction with either **4** or **6**: *Candida cylindracea*, *Mucor miehei*, *Rhizopus arrhizus*, *Rhizopus niveus*, *Aspergillus oryzae*, *Candida lipolytica*, *Mucor javanicus*, *Penicillium roqueforti*, *Rhizomucor miehei*, *Bacillus species*, *Bacillus thermoglucosidasius*, *Candida lipolytica*, *Mucor miehei*, *Saccharomyces cerevisae*, and *Thermoanaerobium brockii*.

Scheme 2. *Fluorogenic Esterase Assay with Enantiomeric Primary Acetates* (R)- *and* (S)-**6**. ①: lipase or esterase, ②: HLDH, NAD^+, ③: BSA, pH > 7.

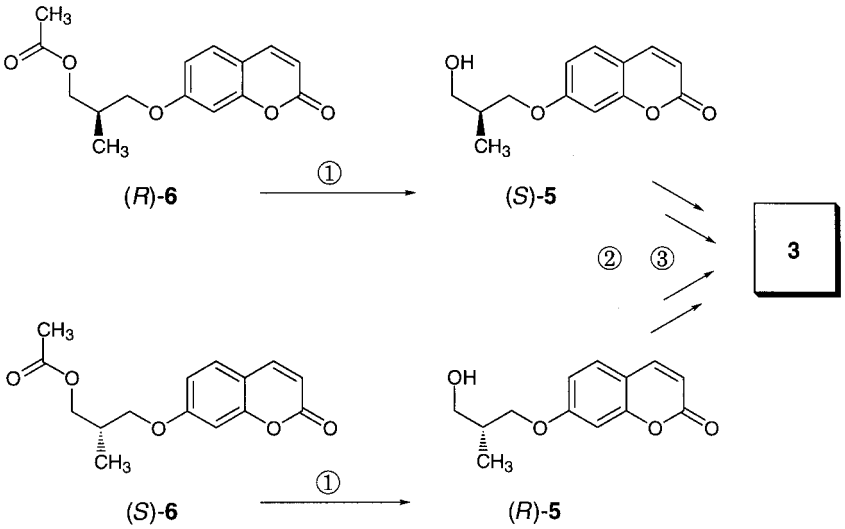

Table 1. *Apparent Rates of Oxidation of Alcohol Substrates by Alcohol Dehydrogenases*[a])

Alcohol dehydrogenase	(*R*)-**1**	(*S*)-**1**	ee [%]	(*R*)-**5**	(*S*)-**5**	ee [%]
Horse liver[b]) (10 μg/ml)	140	2460	89.2 *S*	47	2300	95.6 *S*
Yeast[b]) (100 μg/ml)	3.31	599	98.9 *S*	2.44	7.68	51.8 *S*
Ther. brockii[c]) (10 μg/ml)	12.2	3130	99.2 *S*	0.462	29.5	96.9 *S*

[a]) Apparent rate of release of umbelliferone (**3**) in pM · s^{-1}. Initial rate of release of **3** is reported as calculated from the fluorescence signal according to a calibration curve with pure **3**. [b]) Conditions: 20 mM aq. borate (pH 8.8), 26°, 100 μM substrate, 2 mg/ml of BSA, enzyme, and 1 mM NAD^+. [c]) Conditions: see *Footnote b*, but 1 mM $NADP^+$ instead of $NADP^+$.

To probe whether the fluorescence measurements reflected actual catalytic and kinetic resolution potential by the lipases and esterases, a comparison with preparative conditions was required. A series of eleven representative kinetic resolution experiments were carried out with racemic ester **4** (*Table 3*). Five of the six lipases that showed apparent catalysis of acetate cleavage in the HLDH-coupled fluorogenic assay also promoted acetate hydrolysis under preparative conditions. The sixth was the esterase from wheat germs, which precipitated and did not show any activity in the preparative setup. For the active lipases, a good correlation was observed between lipase reactivity in the fluorogenic assay (V_T in *Table 3*) and the reaction rate under preparative conditions. Finally, five lipases found to be inactive in the HLDH-coupled fluorogenic assay did not show any activity in the preparative setup. Thus, our assay was reliably predicting catalytic activity.

The enantiomeric purity of the alcohol (*R*)-**1** produced by kinetic resolution of acetate **4** under preparative conditions was determined by chiral-phase HPLC.

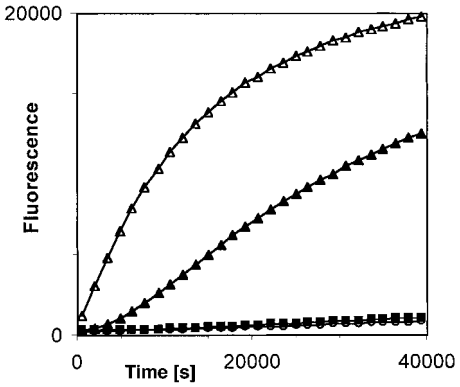

Fig. 1. *Fluorescence signals* (arbitrary units) *observed in 20 mM aq. borate* (pH 8.8) *using* λ_{ex} *360 ± 20 nm and* λ_{em} *460 ± 20 nm with HLDH* (50 μm/g/mL) *and BSA* (2 mg/ml): △, *50* μM (S)-**1** → **3**; ▲, *50* μM (S)-**4** → **3** *with* Pseudomonas *species* B *lipase* (10 μg/ml); ■, *50* μM (S)-**4** → **3** *with* Candida antarctica *lipase* (10 μg/ml); • *100* μM (S)-**4** → **3** *without lipase*. The fluorescence reading at $t = 0$ reflects fluorescence from (*S*)-**4** or (*S*)-**1**, which were completely free of **3** as assessed by HPLC.

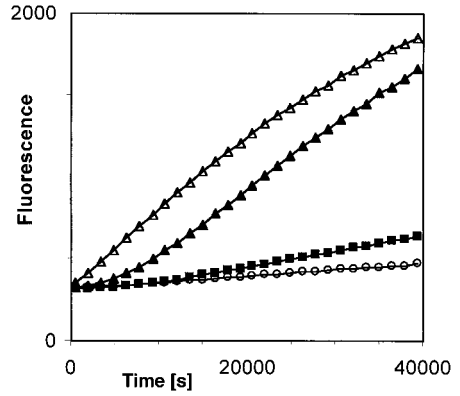

Fig. 2. *Fluorescence signals* (arbitrary units) *observed in 20 mM aq. borate* (pH 8.8) *using* λ_{ex} *360 ± 20 nm and* λ_{em} *460 ± 20 nm with HLDH* (50 μg/ml) *and BSA* (2 mg/ml): △, *50* μM (R)-**1** → **3**; ▲, *50* μM (R)-**4** → **3**; *with* Pseudomonas *species* B *lipase* (10 μg/ml); ■, *50* μM (R)-**4** → **3** *with* Candida antarctica *lipase* (10 μg/ml); •, *100* μM (R)-**4** → **3** *without lipase*. The fluorescence reading at $t = 0$ reflects fluorescence from (*R*)-**4** or (*R*)-**1**, which were completely free of **3** as assessed by HPLC.

Strikingly, the only lipase giving a large enantiomeric ratio E in the fluorescence assay ($E = 26$), namely *Candida antartica* lipase, is also the only one giving a useful enantiomeric excess on preparative scale (100% ee). For most lipases, predicted and observed enantiomeric excesses lie within 20%, which is satisfactory given the assay's complexity. The larger discrepancy between predicted and preparatively observed enantiomeric excess in the case *Pseudomonas fluorescens* lipase might be caused by the occurence of an unusually low K_M for one of the enantiomers. Indeed, the outcome of a kinetic resolution is determined by the enatiomeric ratio $E = (k_{cat}/K_M(R))/(k_{cat}/K_M(S))$, and the apparent catalytic rates measured in the assay are proportional to the specificity constants k_{cat}/K_M only as far as the substrate concentrations lie below K_M,

Table 2. *Fluorogenic Assay for the Kinetic Resolution of Acetates* **4** *and* **6**

Substrate	Lipase	$V(R)$ pM · s^{-1}	$V(S)$ pM · s^{-1}	$V_{rel}(R)$[d])	$V_{rel}(S)$[d])	E[e])	ee[%][f])
4	*Candida antarctica*[a])	4.2	5.6	0.13	0.005	26	93 (*R*)
4	*Pseudomonas* species[a])	4.7	170	0.14	0.15	0.93	3 (*S*)
4	*Ps.* species type *B*[a])	29	390	0.92	0.36	2.6	44 (*R*)
4	*Chromobact. viscosum*[a])	1.6	60	0.051	0.054	0.94	3 (*S*)
4	Wheat germ[a])	5.9	94	0.18	0.085	2.1	37 (*R*)
4	*Pseudomonas fluorescens*[a])	0.57	5.6	0.018	0.005	3.6	56 (*R*)
6	*Candida antarctica*[b])	270	8.2	0.20	0.13	1.5	23 (*R*)
6	*Pseudomonas* species[b])	430	37	0.79	0.18	4.4	62 (*R*)
6	*Ps.* species type *B*[b])	200	7.6	0.24	0.18	1.3	12 (*R*)
6	*Chromobact. viscosum*[b])	220	7.4	0.23	0.21	1.1	4 (*R*)
6	Wheat germ[b])	82	3.1	0.095	0.077	1.2	11 (*R*)
6	*Pseudomonas fluorescens*[b])	68	2.3	0.059	0.032	1.8	30 (*R*)
6	*Aspergillus niger*[c])	250	13	0.28	0.11	2.5	44 (*R*)
6	Hog pancreas[c])	62	3.1	0.066	0.027	2.4	42 (*R*)
6	*Pseudomonas cepacia*[c])	430	19	0.40	0.19	2.1	36 (*R*)
6	*Bacillus stearothermophilus*[b])	110	9.2	0.25	0.071	3.5	56 (*R*)

Conditions: 50 μM (*R*)-**4**, (*S*)-**4**, or 100 μM (*R*)-**6** or (*S*)-**6**, 2 mg/mL BSA, 1mM NAD^+, and [a]) 20 mM aq. borate, pH 8.8, 26°, 50 μg/mL HLDH, 10 mg/ml lipase, or [b]) 20 mM *Bis-tris*, pH 7.2, 26°, 10 μg/ml HLDH, 10 mg/ml or [c]) 100 μg/ml lipase. [d]) $V_{rel}(S) = V(S)/V_{OH}$, $V_{rel}(R) = V(R)/V_{OH}$, V_{OH} = rate observed under the respective assay conditions with 50 μM (*R*)-**1**, (*S*)-**1**, or 100 μM (*R*)-**5**, (*S*)-**5** without lipase. [e]) E = enantiomeric ratio estimated as $E = (V(R)/V_{OH}/V(S)/V_{OH})$. [f]) Predicted enantioselectivity for released alcohol in the initial phase of the reaction. $ee = (V(R)/V_{OH} - V(S)/V_{OH})/(V(R)/V_{OH} + V(S)/V_{OH})$. The following lipases and esterases gave no reaction with either **4** or **6**: *Candida cylindracea, Mucor miehei, Rhizopus arrhizus, Rhizopus niveus, Aspergillus oryzae, Candida lipolytica, Mucor javanicus, Penicillium roqueforti, Rhizomucor miehei, Bacillus* species, *Bacillus thermoglucosidasius, Candida lipolytica, Saccharomyces cerevisae, Thermoanaerobium brockii.*

Table 3. *Lipase Performances Predicted and Observed under Preparative Conditions*

Lipase	V_T[a])	Predicted ee [%][b])	t [h][c])	% conv.	Observed ee [%]
Candida antarctica	0.135	93 *R*	16	36	100 *R*
Pseudomonas species	0.29	3 *S*	2	24	22 *R*
Ps. species type *B*	1.28	44 *R*	1	56	39 *R*
Chromobact. viscosum	0.105	3 *S*	16	42	12 *R*
Wheat germ	0.265	37 *R*	16	0	–
Pseudomonas fluorescens	0.023	56 *R*	16	27	8 *R*
Mucor miehei	0.00	–	24	0	–
Rhizopus arrhizus	0.00	–	24	0	–
Candida lipolytica	0.00	–	24	0	–
Mucor javanicus	0.00	–	24	0	–
Rhizomucor miehei	0.00	–	24	0	–

[a]) $V_T = V(R)V_{OH} + V(S)/V_{OH}$ (see *Table 2*). [b]) As calculated from the assay. [c]) Preparatives conditions are: 10 mg/ml of **4** with 4 mg/ml of lipase in 20 mM aq. borate (pH 8.8), 20% (*v*/*v*) acetonitrile, 26°. Samples were analyzed by chiral-phase HPLC.

under which conditions their ratio approaches the true enantiomeric ratio. Although the low substrate concentrations of our assay (50 – 100 μM) can be expected to be below K_M in most cases, it is probably not the case for *Pseudomonas fluorescens* lipase.

Conclusion. – We have reported a simple and efficient enantioselective fluorogenic assay for the kinetic resolution of chiral acetates with unbiased chemical reactivity. The assay with secondary acetates (*R*)- and (*S*)-**4** also operates in cell-culture media, and can be used to screen libraries of antibodies for enantioselective catalysis of acetate hydrolysis. Primary acetates (*R*)- and (*S*)-**6** are unstable in cell-culture media and can only be used to assay partially purified enzyme preparations.

It should be noted that the intensity of the fluorescence signal produced allows one to use very dilute substrate concentrations. In addition to the advantages discussed above in relation to predicting enantiomeric excesses, low substrate concentrations permit low detection limits of the catalysts to be assayed. Our assay requires only 10 μg/ml of the solid lipase or esterase sample. By comparison, a similar procedure reported using chromogenic nitrophenyl esters required at least 100-fold larger amounts of enzyme (> 1 mg/ml) to achieve detection [7a]. The high sensitivity of our assay provides a key advantage for detecting lipase catalytic antibodies in cell culture, where their concentration is *ca.* 5–50 μg/ml.

Although our assay detects kinetic resolution of relatively simple acetates, the unbiased reactivity of these test substrates should warrant that a novel biocatalyst uncovered by this assay will accept a range of different alkyl acetates as substrates. The need for such novel catalysts is illustrated by the fact that none of a series of thirty commercially available esterases and lipases hydrolyzes acetate (*S*)-**4**, (*R*)-**6**, or (*S*)-**6** enantioselectively.

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Experimental Part

*(*R*)- and (*S*)-7-(3-Hydroxybutoxy)-2*H*-1-benzopyran-2-one* (**1**). A mixture of 7-hydroxy-2*H*-1-benzopyran-2-one (165 mg, 1.0 mmol), NaH (60% suspension in oil; 45 mg, 1.1 mmol) and (*R*)-3-[(tetrahydro-2*H*-pyranyl)oxy]butyl *p*-toluenesulfonate (222 mg, 0.68 mmol) [12] were heated in DMF (4 ml) for 24 h at 60°. The mixture was then diluted with AcOEt and washed successively with H_2O and 1M aq. NaOH. Evaporation of the org. phase and flash chromatography (FC; hexane/AcOEt 3:2; R_f 0.70) gave the tetrahydropyranyl-protected derivative of **1**, which was dissolved in 5% aq. MeOH (5 ml) containing *p*-toluene sulfonic acid (cat.). After 2 h at 25°, the mixture was neutralized with 1 drop of Et_3N. Removal of the solvent and FC (hexane/AcOEt 3:2; R_f 0.23) gave (*R*)-**1** (59 mg, 90%). Colorless oil. $[\alpha]_D^{25} = +8$ ($c = 0.5$, $CHCl_3$). IR ($CHCl_3$): 3433, 2966, 1734, 1706, 1611, 834. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.62 (*d*, *J* = 9.4, 1 H); 7.36 (*d*, *J* = 8.4, 1 H); 6.86 (*d*, *J* = 2.2, 1 H); 6.82 (*dd*, *J* = 8.4, 2.2, 1 H); 6.24 (*d*, *J* = 9.4, 1 H); 4.12 (*m*, 3 H); 2.02 (*m*, 2 H); 1.44 (*d*, *J* = 6.7, 3 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 162.7; 161.9; 156.5; 144.1; 129.4; 113.8; 113.7; 113.3; 102.1; 66.7; 66.1; 38.6; 24.5. Anal. calc. for $C_{13}H_{14}O_4$ (234.25): C 66.65, H 6.02; found: C 66.60, H 5.97.

A similar procedure starting with (*S*)-3-[(tetrahydro-2*H*-pyranyl)oxy]butyl *p*-toluenesulfonate gave (*S*)-**1** as a colorless oil.

*(*R*)- and (*S*)-7-(3-Acetoxybutoxy)-2*H*-1-benzopyran-2-one* (**4**). Acetylation of **1** (Ac_2O/pyridine, 20°, 12 h) followed by co-evaporation of excess reagents with toluene and FC (hexane/AcOEt; R_f 0.53) gave the corresponding acetates **4** in quantitative yields as colorless oils. Anal. data for (*R*)-**4**: $[\alpha]_D^{25} = +24$ ($c = 0.25$, $CHCl_3$). IR ($CHCl_3$): 2929, 1731, 1612, 1250, 907, 728. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.68 (*d*, *J* = 9.4, 1 H); 7.41 (*d*, *J* = 8.4, 1 H); 6.86 (*d*, *J* = 2.2, 1 H); 6.82 (*dd*, *J* = 8.4, 2.2, 1 H); 6.25 (*d*, *J* = 9.4, 1 H); 5.15 (*m*, 1 H); 4.09 (*m*, 2 H); 2.06 (*m*, 5 H); 1.29 (*d*, *J* = 6.7, 3 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 171.3; 162.6; 161.9; 156.5; 144.1; 129.5; 113.8; 113.7; 113.3; 101.9; 68.7; 65.6; 35.9; 21.97; 20.90. Anal. calc. for $C_{15}H_{16}O_5$ (276.29): C 65.21, H 5.84; found: C 65.31, H 5.97.

*(*R*)-and (*S*)-7-(3-Hydroxy-2-methylpropoxy)-2*H*-1-benzopyran-2-one* (**5**). (*S*)-3-Bromo-2-methylpropan-1-ol (300 mg, 1.96 mmol) was added to a mixture of NaH (60% in oil; 103 mg, 2.59 mmol) and 7-hydroxy-2*H*-1-benzopyran-2-one (300 mg, 1.96 mmol) in DMF (2 ml) and heated at 60° for 24 h. The mixture was then diluted

with AcOEt and washed successively with H_2O and 1M aq. NaOH. Evaporation of the org. phase and FC (hexane/AcOEt 3:2; R_f 0.2) gave (*R*)-**5** (298 mg, 67%). Colorless crystalline solid. M.p. 88°. $[\alpha]_D^{25} = +4$ ($c = 1.35$, $CHCl_3$). IR ($CHCl_3$): 3434, 2934, 2254, 1738, 1614, 912, 730. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.62 (*d*, $J = 9.4$, 1 H); 7.36 (*d*, $J = 8.5$, 1 H); 6.84 (*m*, 2 H); 6.24 (*d*, $J = 9.4$, 1 H); 3.99 (*m*, 2 H); 3.70 (*m*, 2 H); 2.24 (*m*, 1 H); 1.08 (*d*, $J = 6.9$, 3 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 162.9; 161.9; 156.5; 144.1; 129.4; 113.7; 113.5; 113.2; 102.1; 71.6; 65.7; 36.2; 14.3. Anal. calc. for $C_{13}H_{14}O_4$ (234.25): C 66.65, H 6.02; found: C 66.65, H 6.01.

A similar procedure starting with (*E*)-3-bromo-2-methylpropan-1-ol (0.3 g, 1.96 mmol) gave (*S*)-**5** (287 mg, 65%) as colorless crystalline solid.

*(*R*)- and (*S*)-7-(3-Acetoxy-2-methylpropoxy)-2*H*-1-benzopyran-2-one* (**6**). Acetylation of **5** as above gave **6** in quantitative yield as colorless solids. Anal. data for (*R*)-**6**: M.p. 51°. $[\alpha]_D^{25} = -2$ ($c = 0.5$, $CHCl_3$). IR ($CHCl_3$): 2972, 2254, 1738, 1614, 1230, 1124, 910, 732, 650. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.62 (*d*, $J = 10$, 1 H); 7.36 (*d*, $J = 8.5$, 1 H); 6.84 (*m*, 2 H); 6.24 (*d*, $J = 8.5$, 1 H); 4.14 (*m*, 2 H); 3.95 (*m*, 2 H); 2.34 (*m*, 1 H); 2.08 (*s*, 3 H); 1.12 (*d*, $J = 15$, 3 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 171.7; 162.7; 161.8; 156.5; 144.1; 129.4; 113.8; 113.6; 113.2; 101.9; 70.6; 66.4; 33.5; 21.5; 14.5. Anal. calc. for $C_{15}H_{16}O_5$ (276.29): C 65.21, H 5.84; found: C 65.21, H 5.86.

Fluorescence Measurements. Substrates were diluted from a 10 mM stock soln. in 50% aq. MeCN. All buffers and solns. were prepared using *MilliQ*-deionized H_2O. Enzymes were diluted from 1 mg/ml stock solns. in PBS (PBS = 10 mM aq. phosphate, 160 mM NaCl, pH 7.4). BSA was diluted from a 40 mg/ml stock soln. in either 20 mM borate (pH 8.8) (assays with **4**) or 20 mM *Bis-tris* (= 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol = {[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]imino}bis[ethanol] (pH 7.2) (assays with **6**). Reactions were initiated by addition of lipase or esterase to a soln. containing substrate, HLDH, and NAD^+.

The 200-µl assays were followed in individual wells of round-bottom polypropylene 96-well plates (*Costar*) with a *Cytofluor-II* fluorescence-plate reader (*Perseptive Biosystems*, filters λ_{ex} 360 ± 20 nm, λ_{em} 460 ± 20 nm). For each assay, fluorescence was converted to umbelliferone (**3**) concentration according to a calibration curve with pure **3** in the same buffers containing BSA. The reference maximum rate with alcohol substrates was taken from the linear portion of the curves (data points 0 to 5000 s). For assays with acetates, the linear portions of the curves (data points 15000 to 30000 s) were used to derive the reaction rates.

Preparative assays: Solid enzyme (1 mg) weighed into a 1.5-ml *Eppendorf* tube was dissolved in 20 mM aq. borate (pH 8.8; 200 µl). Then, a soln. of racemic acetate **4** (50 µl; 50 mg/ml in MeCN) was added and the resulting suspension agitated on a plate stirrer at r.t. To follow the reaction, 2-µl aliquots were taken and diluted with hexane/i-PrOH 1:1. Then, this soln. (20 µl) was injected on a *Chiralpak AS* column (*Daicel*, 0.45 × 22 cm, flow 1.0 ml · min^{-1}, hexane/i-PrOH 1:2): t_R 10.9 ((*R*)-**1**), 23.8 ((*S*)-**1**), 12.1 ((*R*)-**4**), 13.1 ((*S*)-**4**) min. The integral of the peaks recorded by UV at 325 nm was used to calculate the conversion and ee of released alcohol.

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